Growth curve. WT, *pyk1*Δ, *pyk1*Δ + *PYK1*, *pyk1*Δ/*mig1*Δ, and *mig1*Δ strains were inoculated into 10mL YPLR broth and incubated at 30°C with shaking for 24hrs. The cells were then centrifuged, washed once with 10mL sterile PBS, and resuspended in 2.5-5mL sterile PBS for quantification by hemocytometer count. Each strain ($1x10^6$ CFU per strain) was then inoculated into 50mL Yeast Peptone Lactate broth (YPLac; 1% yeast extract, 2% peptone, 2% lactic acid) broth and incubated at 30°C with shaking for 90hrs. Optical density (600nm) was measured for 3 samples (1mL each) per strain at set time-points.

Survival in macrophages. The ability of the *pyk1*Δ and *hxk1*Δ/*hxk2*Δ mutants to survive in macrophages was assessed as previously described (1-3). WT, *pyk1*Δ, *pyk1*Δ + *PYK1*, *pyk1*Δ/*mig1*Δ, *hxk1*Δ, *hxk2*Δ, and *hxk1*Δ/*hxk2*Δ strains were inoculated into YPLR broth and incubated at 30°C for 24hrs with shaking to provide inocula for the macrophage survival assay. All inocula were cultured on YPLac agar plates for quantitative counts of colony forming units to normalize the *C. neoformans* cells in the macrophage killing assay. Briefly, $1x10^5$ activated macrophages (strain J774A.1) were coincubated in a 96-well microtiter plate with $1x10^5$ cells of each *C. neoformans* strain that were opsonized with monoclonal antibody mAb18b7 (a gift from Arturo Casadevall; (2)) at 37°C, 5% CO_2. Following 18-24 hrs of coincubation, the macrophages were lysed with 0.5% SDS. Lysates were serially diluted and plated on YPLac plates for quantitative counts of CFU for each strain. Triplicate cultures were performed for each *C. neoformans* strain.

H_2O_2 and nitric oxide susceptibility testing. *C. neoformans* H99, *pyk1*Δ, *pyk1*Δ + *PYK1*, *pyk1*Δ/*mig1*Δ, *mig1*Δ, *hxk1*Δ, *hxk2*Δ, and *hxk1*Δ/*hxk2*Δ strains were incubated in YPLR broth to saturation at 30°C with shaking to serve as inocula for the H_2O_2 and nitric oxide sensitivity assays. Yeast nitrogen base-lacate-raffinose (YNBLR; 6.7g/L Yeast Nitrogen Base minus amino acids, 2% lactic acid, 1% raffinose) broth was made either buffered at pH4 with 25mM succinic acid (nitric oxide assay) or not buffered (H_2O_2 assay) and filter sterilized. Each *C. neoformans* strain was diluted in the appropriate YNBLR broth (YNBLR for H_2O_2; YNBLR (pH4) for nitric oxide) to $1x10^5$ CFU/mL. 100µL of each strain dilution was inoculated into each well of a 96-well microtiter plate, one row (12 wells) per strain. Sodium nitrite ($NaNO_2$) was used to generate nitric oxide due to the spontaneous generation of nitric oxide from nitrite at acidic pH (4). Serial 2-fold dilutions of either H_2O_2 or $NaNO_2$ were performed in the appropriate YNBLR medium from 16mM to 15.6µM. These dilutions, including a 0mM control, were added to the 96-well plates by column (100µL per well) resulting in a dilution series from 8mM to 7.81µM. The microtiter plates were then incubated at 30°C for 72hrs and viewed. The highest H_2O_2 or $NaNO_2$ concentration allowing growth of the yeast was recorded as the minimum inhibitory concentration (MIC).

Statistical analysis. Unless otherwise noted, all statistical analyses were performed using Microsoft Excel 2008 (Microsoft Corp., Redmond WA) using Student's *t*-test.

References

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